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PRINCIPAL INVESTIGATOR: Karen Liby, M.S.
Nira Ben-Jonathan, Ph.D.

CONTRACTING ORGANIZATION: University of Cincinnati
Cincinnati, Ohio 45267-0553

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13. ABSTRACT (Maximum 200 Words) Prolactin (PRL) is a 23 kDa hormone that targets breast tissue, but its role in breast cancer is controversial. In rodents, PRL promotes spontaneous and carcinogen-induced mammary tumors, while in humans PRL and its receptor are detected in the majority of breast cancer biopsies. We hypothesize that locally produced PRL promotes proliferation and/or survival in breast cancer cells, thus providing a growth advantage for a developing tumor. Exogenous PRL (12-200 ng/ml) significantly increased MDA-MB-435 cell proliferation by 3 and 5 days. PRL also upregulated PRL-R mRNA as determined by RT-PCR and real time PCR. Wild type cells expressed low levels of both PRL-R and PRL mRNA. MDA cells were transfected with 23K or 16K PRL expression vectors and PRL secretion confirmed by a dot blot assay. Clones overexpressing 23K PRL proliferated faster than vector control cells, while clones overexpressing 16K PRL proliferated slower. Stable clones overexpressing 16K PRL or endostatin will be used to determine if 16K hPRL suppresses the growth of breast cancer and metastases in nude mice. Clones overexpressing 23K PRL will be used to determine if PRL is mitogenic and provides a growth advantage for tumors.			
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Introduction

Tumors must induce the formation of new blood vessels in order to grow and metastasize. This change to an angiogenic phenotype follows a disruption in the balance between angiogenic and angiostatic factors produced by the tumor. In breast cancer, a high density of blood vessels is inversely correlated with patient survival. Suppressing tumor growth by targeting its vasculature thus offers a promising therapeutic strategy. The homogeneity and genetic stability of endothelial cells lining blood vessels guard against frequent mutations and the development of drug resistance characteristic of tumor cells targeted by traditional therapeutic approaches. Prolactin (PRL) is a 23 kDa pituitary hormone that has mitogenic, morphogenic, and lactogenic actions on the breast. The role of 23K PRL in breast cancer is controversial, but its N-terminal 16K fragment suppresses proliferation of endothelial cells from several species, inhibits capillary formation in chick embryos, and antagonizes the actions of angiogenic factors. However, the ability of 16K PRL to inhibit tumors *in vivo* has not been tested. The purpose of this thesis work is to test the hypothesis that 16K PRL suppresses angiogenesis *in vivo*. Treatment of breast cancer and metastases with 16K PRL should inhibit tumor vascularization and subsequent growth.

Training

I received a travel award to present a poster at the American Association for Cancer Research Angiogenesis and Cancer Meeting and have presented a poster at the 83rd Annual Meeting of the Endocrine Society in June, 2001. I also have given oral and poster presentations of my research at the University of Cincinnati at the Prolactin Interest Group Retreat, the Cell Biology Student Retreat, and the Graduate Student Research Forum. I also was awarded an Albert J. Ryan Fellowship, which is a competitive fellowship given to select students at the University of Cincinnati, Dartmouth, and Harvard. Other training includes attending weekly seminars sponsored by the Department of Cell Biology and critiquing papers and discussing ongoing research projects in Dr. Ben-Jonathan's laboratory meetings.

Research Objectives and Accomplishments

In the first annual report, the following accomplishments were described: 1) generation and characterization of polyclonal antibodies against prolactin (PRL), 2) development of a rapid dot blot assay to detect PRL, 3) production of recombinant 16K and 23K PRL, 4) establishment of an *in vivo* tumor model, and 5) generation of stable MDA-MB-435 clones over-expressing 16K or 23K human PRL (hPRL).

Objective 1: To confirm the angiostatic activity of 16K PRL *in vitro*.

Ongoing efforts to purify recombinant 16K PRL and confirm its inhibitory activity on endothelial cells were described in the first report. Initially, heparin-purified recombinant 16K PRL appeared to inhibit the proliferation of bovine aortic endothelial cells (BAEC), but additional experiments using several different heparin-purified 16K PRL samples failed to show any inhibition. Possible explanations for these unexpected results include the loss of activity following purification, lack of sensitivity in our endothelial cell assay, or endotoxin contamination in the 16K PRL samples that inhibited endothelial cell proliferation. Independent testing of our 16K PRL samples by Chiron Corporation did reveal some endotoxin contamination. Because of the

conflicting results in the *in vitro* endothelial cell assays and because of the published studies describing the angiostatic activity of recombinant 16K PRL, we decided to proceed with the nude mouse tumor experiments. However, we decided to postpone the studies characterizing 16K PRL receptors in breast tissue until we determine if 16K PRL is anti-angiogenic *in vivo*. Receptor studies with 16K PRL also will be difficult because of an inability to develop a purification protocol that yields a highly purified protein in adequate quantities needed for hormone iodination.

Objective 2: To generate and characterize MDA-MB-435 clones over-expressing 16K or 23K PRL.

MDA-MB-435 cells were transfected with mammalian expression vectors encoding 16K PRL, 23K PRL or an empty vector. These vectors are driven by a CMV promoter and contain the endogenous PRL signal sequence targeting the hormone for secretion. Following selection with puromycin, stable clones were isolated by limiting dilution and then expanded. RT-PCR analysis showed hPRL mRNA expression by these clones (Fig 1), and hormone secretion into the conditioned media (CM) was confirmed by the dot blot assay (Fig 2). Because of the conflicting results with our *in vitro* endothelial assays, a well characterized angiostatic agent was needed as a positive control for the nude mouse experiments. We obtained an endostatin expression plasmid, and generated stable MDA-MB-435 clones overexpressing endostatin. Endostatin production and secretion were confirmed by RT-PCR and a dot blot assay.

While characterizing the growth rates of the clones, we observed that clones overexpressing 23K PRL proliferated faster than vector controls, while clones overexpressing 16K PRL proliferated slower (Fig 3). These unexpected results suggested that 23K PRL was acting as an autocrine/paracrine growth factor and that 16K PRL was acting as a PRL antagonist. The possibility that 16K PRL can act as an antagonist will be tested in experiments described under future directions.

PRL as an autocrine/paracrine growth factor in breast cancer.

The role of 23K PRL in breast cancer has been controversial. In rodents, PRL promotes both spontaneous and carcinogen-induced mammary tumors, but studies attempting to correlate circulating serum PRL levels with the development and progression of human breast tumors are inconclusive. In addition, suppression of serum PRL levels by pharmacological agents was ineffective in treating breast cancer patients with advanced disease. However, PRL and its receptor are detected in over 90% of human breast cancer biopsies, suggesting that the hormone may function as an autocrine/paracrine growth factor.

To test this hypothesis, we incubated wild type MDA-MB-435 human breast cancer cells with exogenous hPRL (12-200 ng/ml) and showed a significant increase in cell proliferation by 3 and 5 days (Fig 4). The same PRL concentrations also increased PRL receptor (PRL-R) mRNA expression as detected by RT-PCR (Fig 5 upper panel) and real time PCR (Fig 5 lower panel). These experiments confirmed that MDA cells express low levels of both PRL and PRL-R mRNA.

Objective 3: To establish immunohistochemistry for PRL and von Willebrand factor.

In order to estimate microvessel density in tumors, we have developed an immunohistochemistry protocol for staining von Willebrand factor, a marker specific for endothelial cells. Prominent staining of blood vessels is observed in MDA-MB-435 tumors, but no staining is observed with the control antibody (Fig 6). Immunohistochemical staining of a tumor from MDA cells overexpressing 23K PRL shows that the tumor is making PRL. No staining is observed with a control antibody.

Future Directions: To test the angiostatic activity of 16K PRL and endostatin *in vivo*

Nude mice will be divided into four groups. The mice will be inoculated with clones overexpressing 16K PRL, 23K PRL, endostatin, or a vector control. Tumor size, proliferative and apoptotic indices, microvessel density, and the number of lymph node and lung metastases will be compared. These studies will allow us to determine whether 16K PRL is angiostatic to endothelial cells or whether it acts as a PRL antagonist to the MDA breast cancer cells. This experiment will also verify whether 23K PRL is mitogenic and provides growth advantage to tumor cells.

Figure 1: Expression of PRL and PRL-R by stably transfected MDA clones

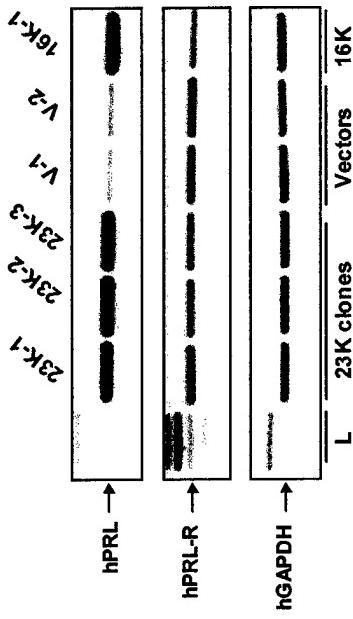


Figure 2: MDA clones secrete PRL as detected by a dot blot assay

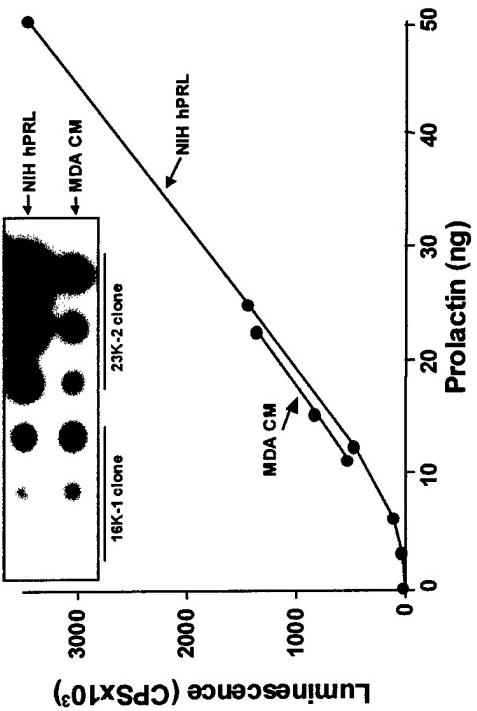


Figure 3: Different growth rates of MDA clones

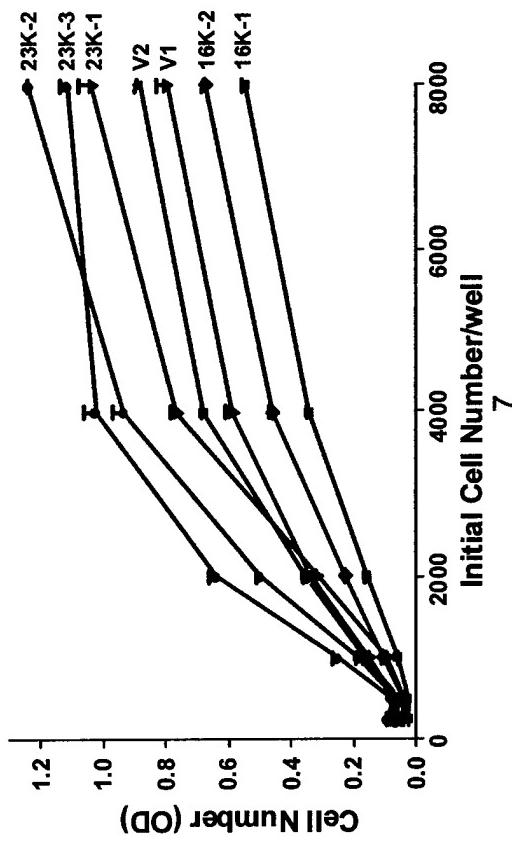


Figure 4: Exogenous hPRL induces MDA-MB-435 cell proliferation

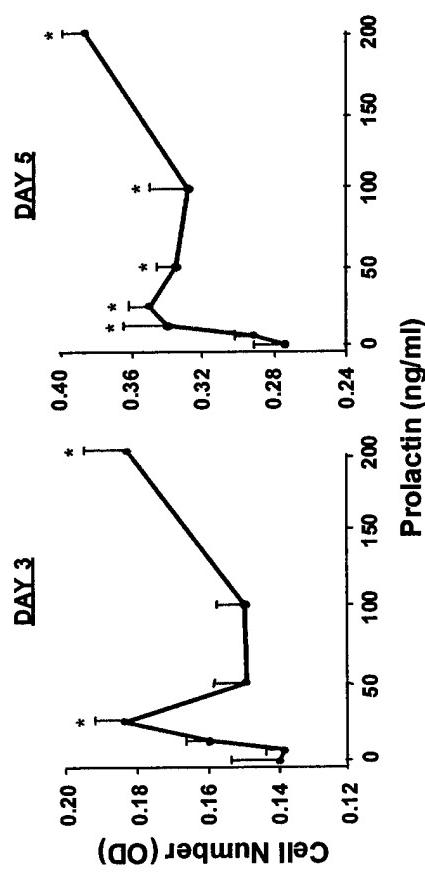


Fig 5: Exogenous PRL upregulates its own receptors

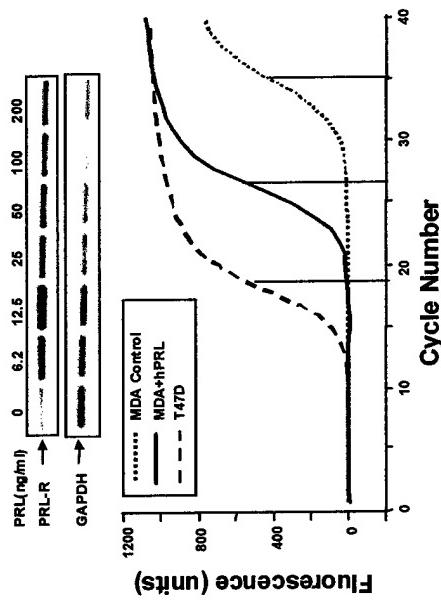
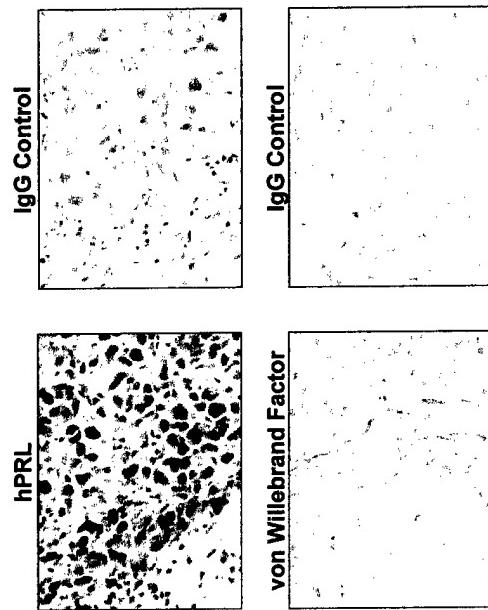


Figure 6: ICC for PRL and von Willebrand Factor



Key Research Accomplishments

- MDA clones that overexpress 16K hPRL, 23K hPRL, endostatin, or vector controls have been generated and protein production and secretion by the clones have been confirmed.
- Protocols for immunohistochemical staining of PRL and von Willebrand factor have been established.
- MDA-MB-435 human breast cancer cells express PRL and PRL-R mRNA.
- Exogenous hPRL increased MDA proliferation and PRL-R mRNA expression.
- MDA clones overexpressing 23K hPRL proliferate faster than vector control clones *in vitro*; clones overexpressing 16K hPRL proliferate slower.

Reportable Outcomes

Publication

Liby K, Neltner B, Mohamet L, Ben-Jonathan N. Prolactin as an autocrine/paracrine growth factor in human breast cancer. In preparation.

Abstracts

Liby K and Ben-Jonathan N. Is 16 kDa prolactin an angiogenic inhibitor in breast cancer? Poster presentation, AACR Special Meeting on Angiogenesis and Cancer, Traverse City, MI; 2000.

Liby K, Neltner B, Mohamet L and Ben-Jonathan N. Prolactin as an autocrine growth factor in human breast cancer. Poster presentation, The 83rd Annual meeting of the Endocrine Society, Denver, CO; 2001.

Conclusions

I have generated MDA-MB-435 clones that overexpress 16K PRL, 23K PRL, endostatin, or vector controls and have established protocols for the immunohistochemical staining of PRL and von Willebrand Factor. Experiments with MDA-MB-435 human breast cancer cells show these cells express mRNA for PRL and the PRL-R and that exogenous PRL induces cell proliferation and increases mRNA expression of the PRL-R. Clones overexpressing 23K PRL proliferate faster than vector controls; clones overexpressing 16K PRL proliferate slower. The MDA clones overexpressing 16K PRL and endostatin will be used to determine if 16K PRL suppresses the growth of tumors and inhibits metastases in nude mice. Clones overexpressing 23K PRL will be used to determine if PRL is mitogenic and provides a growth advantage for tumors.

Abstract for the AACR Special Meeting on Angiogenesis and Cancer

Is 16 kDa prolactin an angiogenic inhibitor in breast cancer?

Karen Liby and Nira Ben-Jonathan. Department of Cell Biology, University of Cincinnati.

Tumors must induce the formation of new blood vessels in order to grow and metastasize. This change to an angiogenic phenotype results from an imbalance between angiogenic and angiostatic factors produced by the tumor. Prolactin (PRL) is a 23 kDa pituitary hormone that has mitogenic, morphogenic, and lactogenic actions on the breast, but its role in breast cancer is unclear. Its N-terminal fragment, named 16K PRL, suppresses endothelial cell proliferation *in vitro* and inhibits capillary formation *in vivo*, but its ability to inhibit tumor growth has not been examined.

The objectives were to: a) produce recombinant human 16K PRL by baculovirus expression, b) generate polyclonal antibodies against hPRL and develop a rapid assay for its detection, c) purify the recombinant PRL and confirm its angiostatic activity, d) establish an *in vivo* tumor model and e) generate mammalian cell lines overexpressing 16K hPRL as a method of hormone delivery *in vivo*.

Baculovirus expression vectors encoding 16K or 23K hPRL were transfected into Sf9 insect cells and PRL secretion was confirmed by Western blotting. After generating highly specific polyclonal antibodies against hPRL, we developed a novel dot blot/chemiluminescence assay that is linear from 3-100 ng of either hormone and takes only 4 hrs. Some preparations of heparin-purified 16K recombinant hPRL inhibited the proliferation of bovine aortic endothelial cells (BAEC) in a dose-dependent manner. The 23K recombinant hPRL had no effect on BAEC, 3T3 or breast cancer cells. Using RT-PCR, we showed that the human breast cancer cell line MDA-MB-435 expresses the angiogenic factors VEGF and FGF-2. Wild type MDA-MB-435 cells were injected into the mammary fatpad of athymic female mice. Tumors developed within 2 weeks, reached a size of 0.5 cm² by 6 weeks, and developed metastases by 12 weeks. Both MDA-MB-435 and insect cells can cleave 23K hPRL into a 16K fragment.

In conclusion, we have produced recombinant 16K and 23K PRL, developed a rapid and sensitive method for their measurement, established an *in vivo* tumor model, and discovered that insect and breast cancer cells can cleave 23K hPRL. We currently are generating stable MDA-MB-435 clones that overexpress 16K or 23K hPRL to determine if 16K PRL suppresses the growth of breast cancer and metastases in nude mice. (Supported by Army grant DAMD17-99-1-9128).

Abstract for the 83rd Annual Meeting of the Endocrine Society

Prolactin as an autocrine growth factor in human breast cancer.

Liby K, Neltner B, Mohamet L and Ben-Jonathan N. Department of Cell Biology, University of Cincinnati.

Prolactin (PRL) is a 23 kDa pituitary hormone that targets the mammary gland, but its role in breast cancer is controversial. In rodents, PRL promotes both spontaneous and carcinogen-induced mammary tumors, while in humans PRL and its receptor are detected in the majority of human breast cancer biopsies. We hypothesize that locally produced PRL promotes cell proliferation and/or survival in breast cancer cells, thus providing a growth advantage for a developing tumor.

We chose MDA-MB-435 human breast cancer cells, which are estrogen- and androgen receptor-negative, and form tumors and metastasize in nude mice. The objectives were to: a) determine the effect of PRL on cell proliferation and PRL receptor (PRL-R) expression, b) generate and characterize MDA-MB-435 clones over-expressing 23K PRL and c) compare the growth of these clones to vector control cells *in vitro* and *in vivo*.

Addition of exogenous PRL (12-200 ng/ml) significantly increased MDA-MB-435 proliferation, but only when the cells were incubated in charcoal stripped serum (CSS) to remove lactogenic hormones from the media. Exogenous PRL also upregulated PRL-R mRNA, as determined by RT-PCR and real-time PCR. Wild type cells expressed low levels of both PRL-R and PRL mRNA. MDA cells were transfected with a 23K human PRL expression vector, and stable clones were selected and expanded. RT-PCR and a dot blot chemiluminescence confirmed PRL production and secretion by these clones. The secreted PRL (0.5-6.5 pg/ml/cell/3days) was biologically active, as confirmed by the rat Nb2 lymphoma proliferation assay. When incubated in CSS, PRL-overexpressing clones proliferated faster than vector control cells *in vitro*. Within four weeks after inoculating nude mice, PRL-overexpressing cells formed significantly larger tumors than vector control cells.

In conclusion, MDA-MB-435 cells express PRL and PRL-R, and exogenous PRL increases cell proliferation and PRL-R expression. Clones over-expressing PRL grow faster than vector control clones both *in vitro* and *in vivo*. These data suggest that locally produced PRL can form a positive autocrine feedback loop resulting in enhanced tumor growth. (Supported by Army grant DAMD17-99-1-9128 and NCI CA80920).